

# siRNA-induced silencing of hypoxia-inducible factor 3 $\alpha$ (*HIF3 $\alpha$* ) increases endurance capacity in rats

**AUTHORS:** Drozdovska S<sup>1</sup>, Gavenauskas B<sup>2</sup>, Drevytska T<sup>2</sup>, Nosar V<sup>2</sup>, Nagibin V<sup>2</sup>, Mankovska I<sup>2</sup>, Dosenko V<sup>2</sup>

<sup>1</sup> National University of Physical Education and Sport of Ukraine;

<sup>2</sup> Department of Hypoxic Research, Department of General and Molecular Pathophysiology, Bogomoletz Institute of Physiology, National Academy of Science, Kiev, Ukraine

**ABSTRACT:** Molecular mechanisms of adaptation to exercise despite a large number of studies remain unclear. One of the crucial factors in this process is hypoxia inducible factor (HIF) that regulates transcription of many target genes encoding proteins that are implicated in molecular adaptation to hypoxia. Experiments were conducted on 24 adult male Fisher rats. Real-time PCR analysis was performed for quantitative evaluation of *Hif3 $\alpha$* , *Igf1*, *Glut-4* and *Pdk-1* in m. gastrocnemius, m. soleus, in lung and heart tissues. Mitochondrial respiratory function and electron microscopy were performed. Knockdown of *Hif3 $\alpha$*  using siRNA increases time of swimming to exhaustion by 1.5 times. Level of mitochondrial NAD- and FAD-dependent oxidative pathways is decreased, however efficiency of phosphorylation is increased after *Hif3 $\alpha$*  siRNA treatment. Expression of HIF target genes in muscles was not changed significantly, except for increasing of *Pdk-1* expression in m. soleus by 2.1 times. More prominent changes were estimated in lung and heart: *Igf1* gene expression was increased by 32.5 and 37.5 times correspondingly. *Glut4* gene expression in lungs was increased from undetected level till 0.3 rel. units and by 84.2 times in heart. Level of *Pdk1* gene expression was increased by 249.2 in lungs and by 35.1 times in hearts, correspondingly. Some destructive changes in muscle tissue were detected in animals with siRNA-inducing silencing of *Hif3 $\alpha$* .

**CITATION:** Drozdovska S, Gavenauskas B, Drevytska T. siRNA-induced silencing of hypoxia-inducible factor 3 $\alpha$  (*HIF3 $\alpha$* ) increases endurance capacity in rats. *Biol Sport*. 2016;33(2):99–106.

Received: 2015-04-20; Reviewed: 2015-06-11; Re-submitted: 2015-08-01; Accepted: 2015-09-07; Published: 2016-03-06.

Corresponding author:  
Svitlana B. Drozdovska  
National University of Physical  
Education and Sport of Ukraine  
E-mail: Sdrozdovska@Gmail.com

**Key words:**  
hypoxia-inducible factor 3 $\alpha$   
endurance capacity  
RNA interference  
muscle  
lung  
heart

## INTRODUCTION

Molecular mechanisms of response to exercise, fitness, performance training or trainability have been studied during the recent decades [1, 2, 3, 4, 5]. To determinate the role of different genetic factors in regulation of skeletal muscle function, researchers applied different methods of genetic engineering and functional genomics. The most frequently used methods are gene knockout (deletion of the gene) [6, 7, 8], chimeric gene construction [9, 10], plasmid constructions [11], etc. RNA-interference (siRNA-induced gene silencing) is a novel method for knockdown of the genes involved in different mechanisms of adaptation to physical exercises [12, 13], but it is still very poorly investigated in sports medicine.

One of the most important genetically determined physical skills having a great influence on performance in different kinds of sports with mostly aerobic pathway of energy supply, is endurance connected with hypoxic response [1]. One of the crucial factors in this process is hypoxia inducible factor (HIF) that regulates transcription of many target genes encoding proteins that are involved in molecular adaptation to hypoxia [7, 14].

The new data about antagonistic effects of different subunits of HIFs that have been obtained recently reveal that some splice vari-

ants of HIF3 $\alpha$  can prevent transcription of target genes of *Hif1 $\alpha$*  and *Hif2 $\alpha$*  [7, 15, 16, 17]. Based on these data, HIF3 $\alpha$  is considered as the negative regulator of HIF1 $\alpha$  and HIF2 $\alpha$ .

In our previous work [12], we have revealed the restricted role of HIF3 $\alpha$  subunit in adaptation to intermittent hypoxia and physical exercise but the mechanisms of this effect remain to be uninvestigated. We hypothesized that *Hif3 $\alpha$*  silencing can lead to increase of the endurance capacity of rats. In our experiments we knocked down *Hif3 $\alpha$*  using siRNA to study rats endurance capacity and established that time of swimming to exhaustion is increased by 1.5 times after the injection of *Hif3 $\alpha$*  siRNA, but the level of NADH- and FAD-dependent oxidative pathways is decreased. The efficiency of phosphorylation and the respiratory control ratio is significantly increased after *Hif3 $\alpha$*  siRNA treatment. Simultaneously, some destructive changes in muscle tissue were detected in animals with RNA-inducing silencing of HIF-3 $\alpha$ .

## MATERIALS AND METHODS

**Animals.** Experiments were conducted on 24 adult male Fisher rats (weight range 200 to 220 g). The animals were housed (4 per cage)

in a room with 12:12-h light-dark cycle at 22°C and were provided with standard rat forage with water ad libitum. All animals were divided into three groups: control (intact animals); endurance training (ET) + scrambled RNA injection; ET + specific *Hif3α* siRNA injection. Each experimental group consisted of 8 rats. All procedures followed the criteria, technical standards and rights applied to animal research. All trials were followed in accordance with the statements of the European Union regarding the handling of experimental animals. This investigation is confirmed to the laws and local ethical committee guidelines for animal research. This study was approved by the Ukrainian National Academy Of Sciences Bogomoletz Institute Of Physiology Biomedical Ethics Committee, Kiev, Ukraine (No5/3 25.11.2013).

### Animal model of swimming training

Rats were exposed to swimming in groups of three in a tank (77 x 38 x 39 cm) filled with water to a height of 31 cm and at 32±1°C with a load (7.0±1.3% of their body weight), which corresponded to 70-75% VO<sub>2</sub> max for one 30-min period each day for 35 days. The functional swimming test on endurance capacity was carried out before the first and second anti-*Hif3α* siRNA injections and 3 days after them. Endurance capacity of rats was assessed by allowing them to swim with a load of 14.0±1.2% of the body weight to exhaustion. The exhaustion was defined as the point when the rats remained below the water surface for 10s and the time to exhaustion was recorded in minutes. Initially, all the animals were randomly divided into 2 groups - a control group (n = 8) and experimental group (n = 16). The animals from experimental group were trained by swimming for 3 weeks, and then were randomly divided into other 2 groups: the first one has been injected with scrambled RNA and the second group - with small interfering anti-*Hif3α* RNA

### RNA interference

A double-stranded scrambled siRNA (sense 5'- UGU UCA GCG AAA UAU AAC CUU -3' and antisense 5'- UUA CAA GUC GCU UUA UAU UGG-3'), also as well as a *Hif3α* siRNA (sense 5'- AGU AUC AUC UGC GUC CAC UUU-3', and antisense 5'- AGU GGA CGC AGA UGA UAC UUU-3') were prepared from corresponding oligonucleotides provided by Metabion (Germany) according to the manufacturer's

instructions. For gene silencing in vivo, these siRNAs were injected (at a dose of 48 µg, twice within a 7-day period) in the tail vein of rats after a three-week swimming course with a load (group 3). The first injection was made on the 21<sup>st</sup> day and the 2<sup>nd</sup> one - one week later. The endurance capacity was detected before the first and second injections and 3 days after them. The effectiveness of RNA-interference was measured using real-time PCR in muscles (m. gastrocnemius and m. soleus).

### RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated using phenol-chloroform extraction after the processing of homogenization with guanidine isothiocyanate (Trizol RNA Prep 100 kit, Russian Federation). RNA concentration was determined with the use of the NanoDrop spectrophotometer ND1000 (NanoDrop Technologies Inc, USA). cDNA was synthesized from 5 µg of total RNA by reverse transcription with 10 mM Tris-HCl (pH 9.0), 5 mM MgCl<sub>2</sub>; 1 mM dNTPs; 20 U Ribo-Lock, Random hexamer primers (0.5 µg·µl<sup>-1</sup>) and 200 U RevertAid H Minus M-MuLV Reverse Transcriptase. PCR was performed using the Applied Biosystems 2700, PerkinElmer, USA

Gene expression of *Hif3α* (Custom TaqMan® Gene Expression Assays) was determined using TaqMan® Gene Expression Assay ("Applied Biosystems", USA). The pairs of forward and reverse primers for genes above mentioned and the TaqMan probes for the target mRNAs were designed based on the rat mRNA sequence by "Applied Biosystems" (USA). Gene expression in each probe was normalized by GAPDH, using TaqMan Rodent GAPDH Control Reagent (VIC™-Probe). The thermal cycles of PCR amplification were followed: initial denaturation step at 95°C for 20 s, followed by treatment at 95°C for 3 s, and at 60°C for 30 s and for 60 cycles using 7500 Fast Real-time PCR ("Applied Biosystems", USA). Analysis of obtained data was carried out with 7500 Fast Real-time PCR Software.

To estimate gene expression of HIF target genes, PCR amplification was performed using 10 µl SYBR Green PCR Master Mix containing 30 pM of each primer (table.1). Final volume of reaction mix was brought up to 20 µl with deionized water. Thermal cycling conditions comprised an initial denaturation and AmpliTaq Gold® DNA polymerase activation step at 95°C for 10 min, followed 50 cycles, with the following dissociation step. Each PCR was running

**TABLE I.** Primer sequences, PCR conditions, product sizes.

Gene, gene bank accession No	Sequence	Anneling T°C	Cycles number	PCR product size
IGF-1 NM_052807.2	5'-TGG ACC CAG TAT GCA GTC TAT G-3' 5'-AGC TGA GAG GAG GAG TTT GA TG-3'	59.5	50	161
GLUT4 M25482.1	5'-CCG GGA CAC TAT ACC CTA TTC A-3' 5'-CCA AGC ACA GCT GAG AAT ACA G-3'	59.5	50	162
PDK1 NM_053826.2	5'-CAG GGT GTG ACT GAA TAC AAG G-3' 5'-GAG ATG CGA CTC ATG TAG AAC C-3'	59.5	50	98

in duplicate. Calculations were performed using the 7500 Fast System SDS software provided. The cycle threshold (CT) was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. We calculated the expression of the target gene relative to the housekeeping gene as the difference between the threshold values of the two genes.

#### Isolation of liver mitochondria

The mitochondria were isolated from liver removed from the rats after decapitation. Liver tissues of all rats were used for mitochondrial preparation. Mitochondria were isolated by differential centrifugation from the liver homogenate according to Doliba *et al* [18]. Briefly, liver samples were excised, weighted and washed in ice-cold buffer. The minced tissue was rinsed clear of blood with cold isolation buffer and homogenized in a glass Potter-Elvehjem homogenizing vessel with a motor-driven Teflon pestle on ice. The isolation medium contained 120 mM KCl, 2 mM K<sub>2</sub>CO<sub>3</sub>, 1 mM EGTA, 10 mM HEPES, pH 7.2. The suspension was then centrifuged for 4 and 6 min at 500 – 800 x g at 2°C. The mitochondrial fraction was obtained by centrifugation of supernatant for 15 min at 6000 x g. Finally, mitochondria were resuspended in the isolation buffer in the concentration 1.0 to 1.5 mg of protein per 1 ml and kept in the tube on ice until polarographic measurements were made. Protein concentration was measured by the method of Lowry *et al* [19] using serum albumin as a standard.

#### Measurement of mitochondrial respiration

Mitochondrial respiratory function was measured in a water-jacketed chamber using a Clark O<sub>2</sub> electrode by the polarographic method of Chance and Williams [20]. To measure mitochondrial oxygen consumption rates at ADP-stimulation, we used a respirometer (Oxygraph+, UK). Mitochondria were added to the respiration chamber containing a total volume 1.0 ml of respiration medium. The incubation medium contained 120 mM KCl, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM K<sub>2</sub>CO<sub>3</sub>, 10 mM HEPES, pH 7.2. Potassium hydroxide (1.0 N) was used to adjust the pH of the medium to 7.4 at 26°C. Succinate (0.35 mM final concentration), glutamate (3 mM final concentration) and malate (2.5 mM final concentration) were used as oxidative substrates. ADP (phosphate acceptor) was administered in concentration 0.2 mM. We investigated the following mitochondrial oxygen consumption parameters: state 2 (oxygen consumption before the addition of ADP), state 3 (oxygen consumption stimulated by ADP), state 4 (oxygen consumption after cessation of ADP phosphorylation), respiratory control ratio (RCR) (a measure of the dependence of the respiratory rate on ATP synthesis is calculated as the ratio between the rate of oxygen consumption during state 3 and state 4), and the efficiency of phosphorylation (ADP/O ratio). ADP/O ratio was calculated by the method of Estabrook [21].

#### Electron microscopy

For electron microscopy m. gastrocnemius and m. soleus were fixed in 2% paraformaldehyde with 2.5% glutaraldehyde in 0.1 M·l<sup>-1</sup>

phosphate buffer (pH 7.4) for 2 h and postfixed in 1% buffered osmium tetroxide. Then tissues were embedded in epoxic resin (Fluka, 45359) after dehydration through graded ethanol. Ultrathin sections, double-stained with uranyl acetate and lead citrate, were examined with an electron microscope. Morphometric determination of quantity, volume (%) and quantitative (10<sup>-2</sup>· $\mu$ m<sup>-3</sup>) density, shear area (10<sup>-2</sup>· $\mu$ m<sup>-2</sup>) of mitochondria and mitochondrial shape factor were performed using «Organel» software.

#### Statistical analysis

Results were statistically treated with ANOVA and presented as Mean $\pm$ SEM. Statistical differences were considered significant if the P value was <0.05 or <0.01 in case of multiply comparisons using a simple Bonferroni correction.

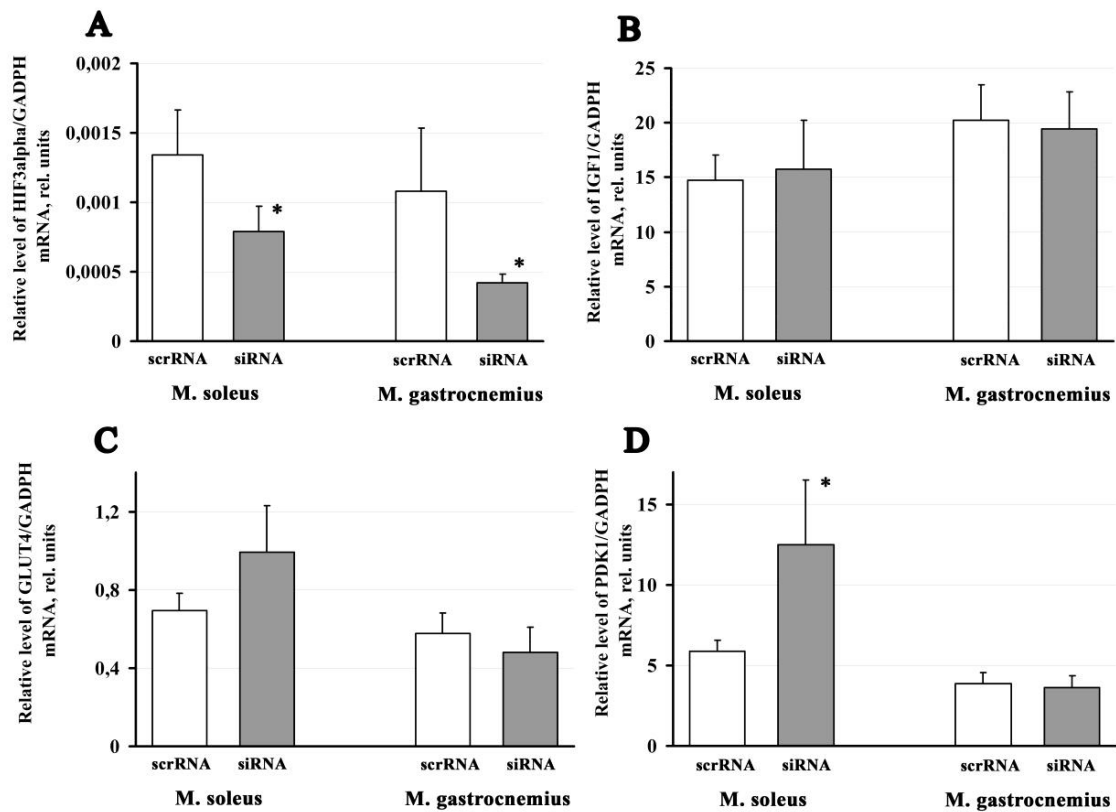
## RESULTS

*siRNA and gene expression.* It was shown that application of interference RNA significantly decreased the level of *Hif3 $\alpha$*  mRNA in both muscles investigated (fig. 1A): by 1.7 times in m. soleus (P<0.03) and 2.6 times in m. gastrocnemius compared to scrambled (P<0.05). Also siRNA significantly decreased the level of *Hif3 $\alpha$*  mRNA in lung and heart - by 2.4 times (P<0.05) and 2.8 times (P<0.05) (fig. 2A). Expression of HIF target genes – *Igf-1*, *Glut-4*, *Pdk-1* was changed at these conditions in a different manner: *Igf-1* mRNA level was not changed, *Glut-4* was increased by 1.4 times in m. soleus (P>0.05), but its level in m. gastrocnemius was not changed, and *Pdk-1* mRNA expression was increased in m. soleus by 2.1 times (P<0.05) and was not changed in m. gastrocnemius compared to scrambled (fig. 1B-D). Conversely, more prominent changes were estimated in lung and heart: *Igf-1* gene expression was increased by 32.5 and 37.5 times correspondingly, compared to scrambled. *Glut-4* gene expression in lungs was increased from undetected level till 0.3 units and in the heart it was increased in 84.2 times. Level of *Pdk-1* gene expression was increased by 249.2 in lungs and in 35.1 times in hearts, correspondingly (fig. 2B-D).

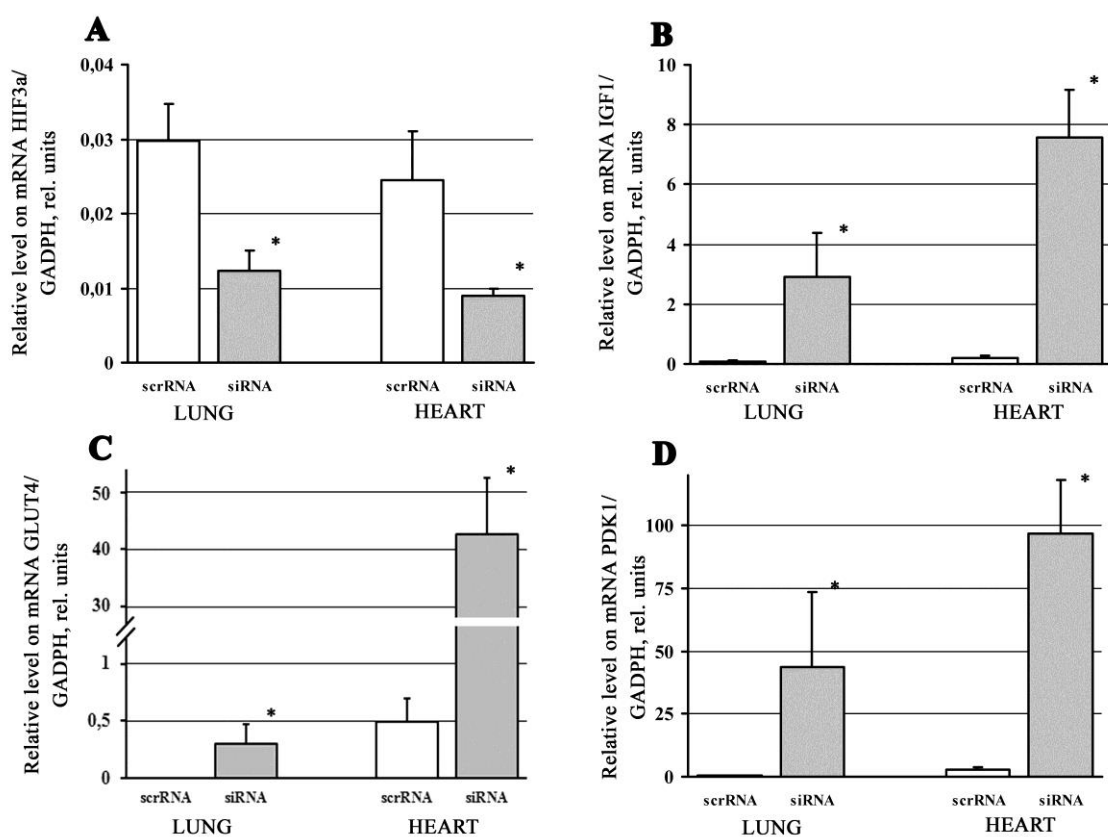
#### Rat endurance capacity at *Hif-3 $\alpha$* silencing

Endurance in control animals was equal to 2.82  $\pm$  0.3 min (fig. 3). The experiment conducted over 3 weeks has shown that swimming training increases the endurance time significantly in 2nd and 3d groups of rats compared to control. There was a significant increase in endurance of animals with ET + scrRNA, against the data obtained in the control group, and its value amounted to 7.67  $\pm$  0.97 min (P<0.05).

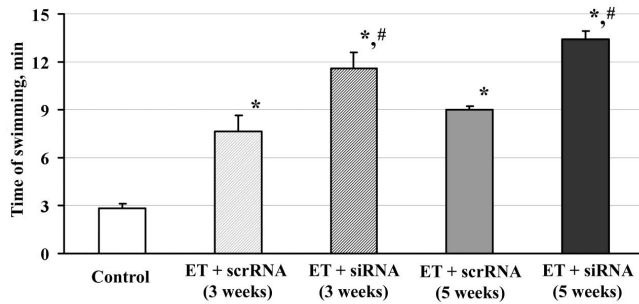
However, more significant endurance increase was observed in animals following the combined endurance training and specific *Hif3 $\alpha$*  siRNA between each other and amounted to 11.59  $\pm$  0.99 min (fig. 3). After 5 weeks of training, we observed the same relationship – the endurance of rats with a siRNA interference was higher than in animals with a scrRNA injection (13.43  $\pm$  0.53 min and 8.98  $\pm$  0.25 min, respectively (P<0.05).



**FIG. 1.** Influence of anti-HIF-3 $\alpha$  siRNA injection on gene expression in soleus and gastrocnemius: A – HIF3 $\alpha$ , B – IGF1, C – GLUT4, D – PDK1. Note: \* – significantly different ( $p < 0.05$ ) compared to ET + scrRNA injection.



**FIG. 2.** Influence of anti-HIF-3 $\alpha$  siRNA injection on gene expression in lung and heart: A – HIF3 $\alpha$ , B – IGF1, C – GLUT4, D – PDK1. \* – significantly different ( $p < 0.05$ ) compared to ET + scrRNA injection.



**FIG. 3.** Endurance (min) in rats with endurance training and siRNA. Control (□), endurance training + scrRNA, 3 weeks (▨), endurance training + siRNA, 3 weeks (▩), endurance training + scrRNA, 5 weeks (■), endurance training + siRNA, 5 weeks (■). Note: \* – significantly different ( $p < 0.05$ ) compared to control, # – significantly different ( $p < 0.05$ ) compared to ET + scrRNA injection.

*Rat mitochondrial function*

The tables 2 and 3 show the respiratory parameters of mitochondria in the liver of rats, using FAD-generating (succinate) and NAD-generating (malate+glutamate) substrates. Endurance training do not influence the rate of oxygen consumption in State 3 and 4, the respiratory control ratio (RCR) and the efficiency of phosphorylation (ADP/O) at succinate and malate+glutamate oxidation in comparison with control group (tables 2 and 3).

**TABLE 2.** Changes in indices of ADP-stimulated mitochondria respiration (substrate of oxidation – 0.35 mM succinate) in liver under endurance training with injections of siRNA.

Animal groups	State 2	State 3	State 4	Respiratory control ratio, RCR	ADP/O, $\mu\text{M ADP} \cdot \text{ng}^{-1}$ at O
	ng at $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein				
Control (n=8)	19.14 $\pm$ 1.57	47.85 $\pm$ 1.42	20.16 $\pm$ 1.50	2.37 $\pm$ 0.13	1.57 $\pm$ 0.10
Endurance training + scrambled siRNA (n=8)	18.94 $\pm$ 1.45	43.86 $\pm$ 2.25	18.18 $\pm$ 1.28	2.42 $\pm$ 0.13	1.68 $\pm$ 0.12
Endurance training + antiHIF3 $\alpha$ siRNA (n=8)	15.84 $\pm$ 1.65 *	40.12 $\pm$ 2.28 *	15.02 $\pm$ 1.11*	2.68 $\pm$ 0.12	1.79 $\pm$ 0.11*

Note: \* – significantly different ( $P < 0.05$ ) compared to control group in tables 2 and 3.

**TABLE 3.** Changes in indices of ADP-stimulated mitochondria respiration (substrate of oxidation – 3 mM glutamate + 2.5 mM malate) in liver under adaptation endurance training with injections of siRNA.

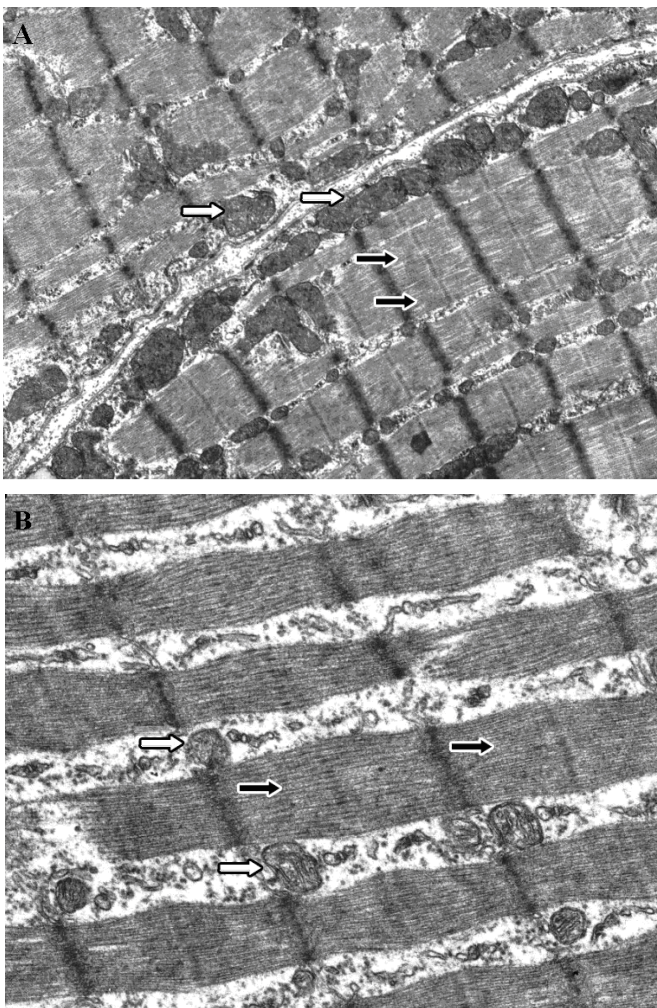
Animal groups	State 2	State 3	State 4	Respiratory control ratio, RCR	ADP/O, $\mu\text{M ADP} \cdot \text{ng}^{-1}$ at O
	ng at $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein				
Control (n=8)	19.20 $\pm$ 1.14	44.90 $\pm$ 1.87	17.81 $\pm$ 1.46	2.54 $\pm$ 0.12	2.34 $\pm$ 0.09
Endurance training + scrambled siRNA (n=8)	17.87 $\pm$ 1.84	46.87 $\pm$ 2.06	17.0 $\pm$ 1.02	2.76 $\pm$ 0.09	2.58 $\pm$ 0.12
Endurance training + antiHIF3 $\alpha$ siRNA (n=8)	14.88 $\pm$ 1.95 *	35.0 $\pm$ 2.98 *	14.77 $\pm$ 1.21 *	2.35 $\pm$ 0.11	2.19 $\pm$ 0.10 *

Combination endurance training and injection of *Hif3 $\alpha$*  siRNA under oxidation of succinate and malate+glutamate has shown the significant decrease of oxygen consumption in States 2, 3, 4 compared to control. At the same time, succinate oxidation increased the efficiency of phosphorylation (by 14%,  $p < 0.05$ ) (table 2), while the oxidation of NAD-dependent substrates did not change these parameters significantly in reference to the control group that also points out the preservation of a high level of the coupling of preceding mitochondrial respiration.

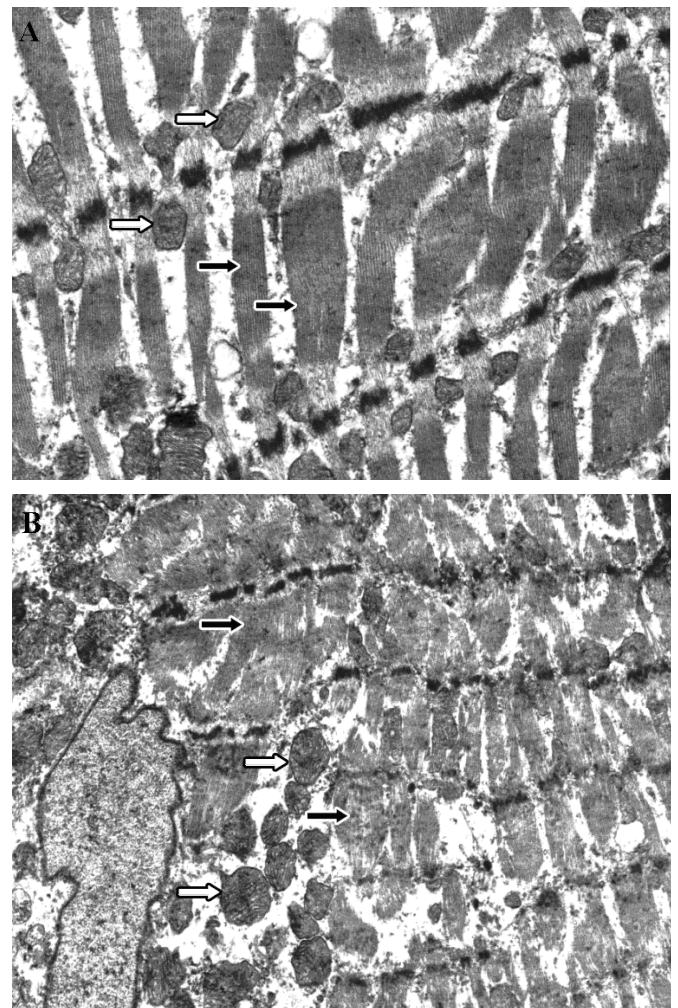
Thus, the injection of siRNA leads to preservation of increase of the work of respiratory chain efficiency against the background of electron transfer function decrease that may indicate the economization of ATP production in liver mitochondria.

*Electron microscopy data*

The interference of *Hif3 $\alpha$*  caused significant changes of m. gastrocnemius ultrastructure to exercise comparing with ET experimental group (fig. 4A, B). The animals with knockdown of *Hif3 $\alpha$*  after endurance training were characterized by heterogeneous ultrastructure of muscle fibers that was represented as alternation of the typical sarcomere sites and reduction of the sarcomeres length in some sites that is a sign of high contractility (fig. 5A, B). Also sites with elongated sarcomeres were detected that might be the indicator of sarcomere dilatation. Myofibrils also underwent structural changes. These changes included both local disjunctions, lyses and varied in intensity of expression. In some sarcomeres lyses covered only separate myofibrils, in other sarcomeres destruction covered I-discs and



**FIG. 4.** Ultrastructure of gastrocnemius (myofibrils – black arrows, mitochondria – white arrows) in rats after continuous physical endurance training with scrRNA injection. A – X12.000; B – X20.000.



**FIG. 5.** Ultrastructure of gastrocnemius (myofibrils – black arrows, mitochondria – white arrows, myofibril destruction – in square) in rats after physical endurance exercises with siRNA injection. A – X19.000; B – X12.000

H-discs or even whole sarcomeres. This resulted in the loss of integrity of the myofibril clump (see fig. 5A, B).

The morphometric analysis proved that volume density, quantitative density and shear area of mitochondria of m. gastrocnemius in ET + specific *Hif3α* siRNA group are significantly lower than in ET + scrRNA group and restore to the control area (table 4). The retaining of quantitative density of mitochondria at the control level after *Hif3α* gene silencing was accompanied by hypertrophy of mitochondria. There was a considerable difference from the intact animals too. The greatest change of size occurred in mitochondria located peripherally – section area was equal to  $36.51 \pm 1.99 \cdot 10^{-2} \cdot \mu\text{m}^2$ . In central sites the average area of mitochondria totaled in  $26.03 \pm 1.92 \cdot 10^{-2} \cdot \mu\text{m}^2$ . Generally, this parameter equals to  $31.27 \pm 1.06 \cdot 10^{-2} \cdot \mu\text{m}^2$ , and the volumetric density value – to  $8.66 \pm 1.52 \%$ . It is worthy to mention here that the symplasts of animals from ET+siRNA group against the background of physical exertion did not contain mitochondria with destroyed external membrane.

The RNA interference of *Hif3α* in the m. soleus against the background of physical exertion caused impairment of the mosaic struc-

ture of symplasts that was marked for its own specific features. The m. soleus, where the number of symplasts is higher comparing to the m. gastrocnemius, contained myofibrils with no signs of expressed destructive changes. Such changes mainly occurred through minor disjunction of myofibrils. At the same time, certain fibers with considerable destructive changes of sarcomeres also occurred, and occasionally spread throughout an entire symplast. This was different from the m. gastrocnemius, where the morphological signs of anomalies in contractility and destruction of myofibrils were limited to separate sites only.

Comparing to the ET + scrRNA group, m. soleus of animals after interference had mitochondria with a high electron density matrix that contained a minor number of crista. Organelles had a small size and (mainly) awkward and slightly prolate shape resulting from invaginations of the external membrane. This was proved by the morphometric analysis: the shape factor is  $0.68 \pm 0.1$ , i.e. lower than the same value in the control group ( $0.80 \pm 0.1$ ) and the value registered after physical exercise ( $0.73 \pm 0.1$ ). Similar to the case with physical load, mitochondria with awkward and more prolate

shape were located in the central sites of symplasts; it means that the *Hif3 $\alpha$*  gene silencing causes the mitochondria to prolapse and awkward shape under long-time physical exertion. Such physical exertion can cause an increase of the mitochondria surface area under conditions of heavy functioning. At that, the size of mitochondria, i.e. their section area, slightly increased (primarily in the central sites) comparing to the same value in the control group (table 3). Moreover, the quantitative density of organelles was statistically lower than in control group. So, a simultaneous increase of size and reduction of number of mitochondria maintains the volume occupied by mitochondria in the cytoplasm at the same level as in control. On the other hand, the quantitative characteristics of mitochondria differed considerably in ET and ET+siRNA groups (see table 3). The *Hif3 $\alpha$*  gene silencing precluded development of mitochondrial hypertrophy that was observed after physical exertion at the peripheral and interfibrillar sites mainly throughout the entire symplast. The average value of section area and the distribution of mitochondria according to this value differed considerably in the groups compared.

**DISCUSSION**

Thus, this study confirms the previously established role of HIF3 $\alpha$  as the negative regulator of other HIF subunits [22, 23]. In our study, we have used siRNA for the first time to silence *Hif3 $\alpha$*  that led to the significant increase of endurance capacity of rats. Optimization of oxidative phosphorylation in mitochondria and increase of HIF dependent gene (*Pdk-1*) expression explain this effect. It is known that PDK-1 due to phosphorylation of PDH (pyruvate dehydrogenase kinase) reduces the flow of pyruvate into the mitochondria and as a result decreases the formation of free radicals [24]. In addition, as we have shown in our previous article [12] physical exercises significantly increase the expression of all alpha subunits of HIF (1 $\alpha$ , 2 $\alpha$ , 3 $\alpha$ ). It should be noted that the level of *Hif3 $\alpha$*  expression lower than the expression level of other two HIF subunits (1 $\alpha$  and 2 $\alpha$ ) up to 50 times. These data are consistent with the results of Hoppeler and Vogt, who have identified elevated *Hif1 $\alpha$*  mRNA concentration in athletes after both high- and low-intensity training under hypoxic conditions that is accompanied with the increase of VEGF, myoglo-

bin and some glycolytic enzymes expression [25, 26]. Faiss [27] have also shown the significant increase of *Hif1 $\alpha$*  mRNA level in the muscle of sprinters after loading training, that is combined with increased expression of carbonic anhydrase III and monocarboxylate transporter-4 mRNA and decreased mRNA expression of mitochondrial transcription factor A, peroxisome proliferator-activated receptor gamma coactivator 1 $\alpha$  and monocarboxylate transporter-1.

However, besides the beneficial effects, siRNA-induced silencing of *Hif3 $\alpha$*  causes destructive changes in the muscle fiber ultrastructure. At the same time, there was significant difference comparing to the group of animals in which the endurance training evoked mitochondrial hypertrophy. RNA-interference causes destructive alteration in muscle fibers of gastrocnemius muscle – lysis and local separation of myofibrils. Also, the changes of the multitude parameters of oxidative phosphorylation measured in isolated mitochondria cannot be definitely interpreted as beneficial. For example, under endurance training with injections of siRNA oxygen consumption in state 2, 3, 4 (using both succinate and glutamate with malate as a substrate for oxidation) in liver was decreased. So, these results seem contra-intuitive, because rats with silencing of *Hif3 $\alpha$*  demonstrate very high response on endurance training. This effect might be related not only with changes in gene expression profile in the muscles, but also could be the result of decreased expression of *Hif3 $\alpha$*  in other organs (lung, heart, liver, kidneys) at systemic siRNA application and increasing of expression of *Hif1 $\alpha$*  and *Hif2 $\alpha$*  target genes. According to some studies, changes in HIFs and its target genes expression are very important in the adaptation of the organism to hypoxia, especially during exercise [3, 4, 12, 24]. Accordingly, Marini has shown increase of mRNA levels of HIFs and other markers of angiogenesis in the heart of trained rats [28]. Ishikawa has found a significant increase of the HIF-1 $\alpha$  protein level in the kidney at the physical exercises that is combined with the improvement of renal function in diabetic rats [29].

Our data indicate that HIFs target genes (*Igf1*, *Glut4* and *Pdk1*) expression levels were dramatically upregulated in lungs and heart as a result of *Hif3 $\alpha$*  silencing. We suggest these changes can explain increase of endurance capacity in rats.

**TABLE 4.** Morphometric mitochondria indices of rat muscles.

Muscle	Animal groups	Volume density of mitochondria, %	Quantitative density, MT, 10 <sup>-2</sup> · $\mu$ m <sup>3</sup>	Shear area, MT, 10 <sup>-2</sup> · $\mu$ m <sup>2</sup>	Shape factor
Gastrocnemius	Control (n=8)	3.37±0.57	22.2±5.18	15.93±0.67	0.83±0.01
	ET+scrRNA (n=8)	27.37±7.17 *	93.19±16.03 *	43.68±1.88 *	0.82±0.01
	ET+siRNA (n=8)	8.66±1.52 #	29.63±2.36 #	31.27±1.06 #	0.81±0.01
Soleus	Control (n=8)	3.72±0.79	27.29±0.59	16.03±1.06	0.80±0.1
	ET+scrRNA (n=8)	17.49±7.07 *	29.44±9.27	42.49±2.54 *	0.78±0.01
	ET+siRNA (n=8)	2.60±0.75 #	16.37±2.59	22.15±1.63 #	0.68±0.1

Note: \* – significantly different (P<0.05) compared to control group; # – significantly different (P<0.05) compared to ET +scrRNA group

Obtained data indicate beneficial effect of *Hif3 $\alpha$*  silencing on endurance capacity in rats and substantiate previous results concerning negative effect of HIF3 $\alpha$  in adaptation to hypoxia. siRNA-induced knockdown of this gene can be used in future studies of the exercise performance mechanisms.

**Conflict of interests:** the authors declared no conflict of interests regarding the publication of this manuscript.

## REFERENCES

- Bouchard C, Malina RM. Genetics of physiological fitness and motor performance. *Exerc Sport Sci Rev*. 1983;11:306-39.
- Bray MS, Hagberg JM, Pérusse L, Rankinen T, Roth SM, Wolfarth B, Bouchard C. The human gene map for performance and health-related fitness phenotypes: the 2006-2007 update. *Med Sci Sports Exerc*. 2009;41:35-73.
- Rankinen T, Roth SM, Bray MS, Loos R, Pérusse L, Wolfarth B *et al*. Advances in Exercise, Fitness, and Performance Genomics. *Med Sci Sp Ex*. 2010;42:835-846.
- Havenauskas BL, Man'kovs'ka IM, Nosar VI, Nazarenko AI, Bratus' LV. Effect of intermittent hypoxic training on indices of adaptation to hypoxia in rats during physical exertion. *Fiziol Zh*. 2004;50:32-42.
- Hagberg JM, Rankinen T, Loos RJ, Pérusse L, Roth SM, Wolfarth B, Bouchard C. Advances in exercise, fitness, and performance genomics in 2010. *Med Sci Sports Exerc*. 2011;43:743-752.
- Aragonés J, Schneider M, Van Geyte K, Aragonés J, Schneider M, Van Geyte K, *et al*. Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nature Genet*. 2008;40:170-180.
- Maynard MA, Evans AJ, Hosomi T, Hara S, Jewett MA, Ohh M. Human HIF-3 $\alpha$ 4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. *FASEB J*. 2005;19:1396-1406.
- Seth A, Steel JH, Nichol D, Pocock V, Kumaran MK, Fritah A, Mobberley M *et al*. The transcriptional corepressor RIP 140 Regulates Oxidative Metabolism in Skeletal Muscle. *Cell Metab*. 2007;6:236-245.
- Arany Z, Spiegelman BM. PGC-1 and metabolic control in skeletal and cardiac muscle. *NC coregulators and human diseases*. *Wold Scientific*. 2008;301-318.
- Hakimi P, Yang J, Casadesus G, Massillon D, Tolentino-Silva F, Nye CK *et al*. Overexpression of the Cytosolic Form of Phosphoenolpyruvate Carboxykinase (GTP) in Skeletal Muscle Repatterns Energy Metabolism in the Mouse. *J Biol Chem*. 2007;282:32844-32855.
- Jang MS, Park JE, Lee JA, Park SG, Myung PK, Lee DH *et al*. Binding and regulation of hypoxia-inducible factor-1 by the inhibitory PAS proteins. *Bioch Bioph Res Comm*. 2005;337:209-215.
- Drevytska T, Gavenauskas B, Drozdovska S, Nosar V, Dosenko V, Mankovska I. HIF-3 mRNA expression changes in different tissues and their role in adaptation to intermittent hypoxia and physical exercise. *Pathophysiology*. 2012;19: 205-14.
- Lee CY, Hu SY, Gong HY, Chen MH, Lu JK, Wu JL. Suppression of myostatin with vector-based RNA interference causes a double-muscle effect in transgenic zebrafish. *Bioch Bioph Res Comm*. 2009;387:766-771.
- Mason SD, Howlett RA, Kim MJ, Olfert IM, Hogan MC, McNulty W *et al*. Loss of Skeletal Muscle HIF-3 results in Altered Exercise Endurance. *Plos Biology*. 2004;10:1540-1547
- Hara S, Hamada J, Kobayashi C, Kondo Y, Imura N. Expression and characterization of hypoxia-inducible factor (HIF)-3 $\alpha$  in human kidney: suppression of HIF-mediated gene expression by HIF-3 $\alpha$ . *Bioch Bioph Res Comm*. 2001;287:808-813.
- Murphy BJ. Regulation of malignant progression by the hypoxia-sensitive transcription factors HIF-1 $\alpha$  and MTF-1. *Comp Bioch Physiol Biochem Mol Biol*. 2004;139:495-507.
- Tanaka T, Wiesener M, Bernhardt W, Eckardt KU, Warnecke C. The human HIF (Hypoxia-inducible factor)-3 gene is a HIF-1 target gene and may modulate hypoxic gene induction. *Biochem J*. 2009;424:143-151.
- Doliba MM, Kurhaliuk NM, Muzyka FV, Shostakovska IV, Kondrashova MM. Effect of alpha-ketoglutarate and acetylcholine synergism on energy metabolism in mitochondria. *Fiziol Zh*. 1993;39:65-70.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:265-275.
- Chance B, Williams GR. The respiratory chain and oxidative phosphorylation. *Adv Enzymol Relat Subj Biochem*. 1956;17:65-134.
- Estabrook RW. Mitochondrial Respiratory Control and the Polarographic Measurement of ADP: O Ratios. *Methods Enzymol*. 1967;10:41-47.
- Li QF, Wang XR, Yang YW, Lin H. Hypoxia upregulates hypoxia inducible factor (HIF)-3 $\alpha$  expression in lung epithelial cells: characterization and comparison with HIF-1 $\alpha$ . *Cell Res*. 2006;16:548-58.
- Isoe T, Hosono O, Tanaka H, Kanopka A, Poellinger L, Haneda M *et al* (2007). Transcriptional up-regulation of inhibitory PAS domain protein gene expression by hypoxia-inducible factor 1 (HIF-1): a negative feedback regulatory circuit in HIF-1-mediated signalling in hypoxic cells. *J Biol Chem*. 2007;282:14073-82.
- Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab*. 2006;3:187-97.
- Hoppeler H, Vogt M. Muscle tissue adaptations to hypoxia. *J Exp Biol*. 2001; 204:3133-9.
- Vogt M, Puntschart A, Geiser J, Zuleger C, Billeter R, Hoppeler H. Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J Appl Physiol*. 2001; 91:173-82.
- Faiss R, Léger B, Vesin JM, Fournier PE, Eggel Y, Dériaz O, Millet GP. Significant molecular and systemic adaptations after repeated sprint training in hypoxia. *PLoS One*. 2013;8:e56522.
- Marini M, Falcieri E, Margonato V, Tréré D, Lapalombella R, di Tullio S, Marchionni C *et al*. Partial persistence of exercise-induced myocardial angiogenesis following 4-week detraining in the rat. *Histochem Cell Biol*. 2008;129:479-87.
- Ishikawa Y, Gohda T, Tanimoto M, Omote K, Furukawa M, Yamaguchi S *et al*. Effect of exercise on kidney function, oxidative stress, and inflammation in type 2 diabetic KK-A(y) mice. *Exp Diabetes Res*. 2012;Article ID 702948, 10 pages.